RESPONSE

Claims 1-17 and 19-24 are currently pending in the above-identified patent application. No claim is allowed.

The Claimed Invention

The claimed invention is a way to induce neuronal progenitor cells to become dopaminergic (DA) cells, that is, to produce dopamine. The way this is done is through adding chemicals to the cells which cause the cells to produce different proteins. In one embodiment, the claimed method starts with NT2/D1 cells that lack a protein, which is an indicator of neuronal cell differentiation. The NT2/D1 cells are treated with an agent such as retinoic acid, which causes some of the cells to express tyrosine hydroxylase, as indicated in Figure 1 of the patent application. Other cells do not change and are called accessory cells. Then the induced cell culture is exposed to mitotic inhibitors such as cytosine arabinoside and fluorodeoxyuridine for several days, during which the DA cells continue to mature and the accessory cells stop dividing and die off. Then the DA cells may be harvested. The claimed method does not genetically alter or transform the cells.

The DA cells also have the other cellular attributes necessary to regulate dopamine. Specifically the D2 dopamine receptor (D2) regulates dopamine release, the dopamine membrane transporter (DAT) is responsible for reuptake of dopamine from the synaptic cleft, and aldehyde dehydrogenase (AHD2) has been found in a subpopulation of dopaminergic neurons naturally found in the mesostriatal and mesolimbic areas, which also are involved in Parkinson's disease. Table 1 of the specification (page 18) shows that all three proteins plus tyrosine hydroxylase are present in the DA neurons.

The specification goes on to exemplify the use of the DA neurons in mammalian research. Example 12 (pages 28-32) shows the successful use of DA neurons in the rat model of Parkinson's disease. Example 12 provides details on how to use the DA neurons in this use, including neurosurgical coordinates for cell placement and specific dosages. The results indicate that the DA neurons were indeed implanted in the desired locations and that dopamine-producing cells could still be detected six-weeks post transplant. "In 43% of animals with DA neuron grafts (n=4), TH+ cells were readily identified in both the striatum and SN." (page 31, lines 16-17) As a group, the DA-implanted rats also had somewhat improved rotational scores (though not statistically significant), an indication of functional improvement. "The lack of significant functional recovery most likely relates to the low number of TH+ neurons, as it has previously been demonstrated that the number of TH+ neurons and fiber outgrowth strongly correlates with the extent of functional recovery." (page 31, line 29, to page 32, line 1)

Baker et al (J Comp Neurol 2000 Oct 9; 426(1):106-16), who reported the results of the above study in a peer-reviewed journal, reported the following: "There was a correlation between surviving THir [TH immunoreactive] cells and rotational scores. Only animals that had surviving

THir cells (43% of the hNT-DA group and [100% of] the LiCl pretreated hNT-DA group) had decreased rotational scores while animals with no THir cells (hNT-neuronal grafts and lesion only groups) did not exhibit any reduction in mean full body turns (Fig. 6)." (pages 110-111)

Section 112, Paragraph 1 Rejection

The Office Action stated that the specification teaches that the only use for the method is to produce cells for gene therapy. In the interview, the Examiner indicated that the term "progenitor cell" could encompass genetically engineered cells and suggested "neuronal progenitor cells." Applicants have amended the claims to recite "neuronal progenitor cells." This rejection on the nonenablement of gene therapy may be withdrawn.

In the Interview, the Examiner also requested that "inhibitor" be changed to "mitotic inhibitor", which the Applicants have done. Therefore, this ground for rejection may be withdrawn.

Also in the interview, the examiner requested that claim 1, step c be changed to "enrich for dopaminergic cells in the culture." Applicants have so amended the claim and this ground for rejection may be withdrawn.

The Office Action stated that claims 1-17 and 19-22 stand rejected because the specification did not enable one skilled in the art to make and/or use the claimed invention. The Office Action stated that methods of transplantation of neural tissue are not routinely successful. In a telephonic interview, the Examiner expanded on this ground for rejection, indicating that the clinical data for function was not significant and requesting further data or correlation. In the working example (Example 12), there are a number of helpful teachings to those skilled in the art, including the number of cells to inject, the site of injection and cellular persistence, as required by the Examiner.
As further explanatory data, the undersigned offers the peer-reviewed journal article Baker et al., EXPERIMENTAL NEUROLOGY 162:350-360, 2000) based on the same study as disclosed in Example 12. According to Baker et al., "[t]here was a correlation between surviving THir cells and rotational scores. Only animals that had surviving THir cells (43% of the hNT-DA group and [100% of]the LiCl pretreated hNT-DA) had decreased rotational scores while animals with no THir cells (hNT-neuronal grafts and lesion-only groups) did not exhibit any reduction in mean full body turns." Thus, a correlation has been established between the presence of surviving dopaminergic cells and functional recovery. Therefore, this ground for rejection may be withdrawn.

Conclusion

Applicants believe that all grounds for rejection have been overcome and a notice of allowance can be issued. To resolve any remaining issues, the Examiner is cordially invited to telephone the undersigned.

Respectfully submitted,
SIERRA PATENT GROUP, LTD.

Dated: May 28, 2002

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Claims as Amended

1. (amended) A method of producing dopaminergic neuronal cells [suitable for transplantation in dopamine deficiencies], said [transplantable] neuronal cells being derived from neuronal progenitor cells,

a. providing neuronal progenitor cells which lack at least one indicator of neuronal cell differentiation;

b. treating the neuronal progenitor cells with an inducing agent for a time period sufficient to optimize expression of tyrosine hydroxylase and to induce the presence of at least one indicator of neuronal cell differentiation to produce a plurality of dopaminergic, differentiated neuronal cells; and

c. minimally replating with [an] a mitotic inhibitor to enrich for dopaminergic cells in the culture [optimize the dopaminergic phenotype and a purified harvest; and

d. harvesting the dopaminergic, differentiated neuronal cells].

11. (amended) A dopaminergic neuronal cell [suitable for transplantation into an individual having a dopaminergic deficiency], said cell comprising

a post-mitotic differentiated neuronal cell which expresses tyrosine hydroxylase and at least one other indicator of neuronal cell differentiation, said cell having undergone induction ex vivo from an undifferentiated neuronal progenitor cell.

12. (amended) A human post-mitotic dopaminergic cell [suitable for transplantation into a human having a dopaminergic deficiency], said cell comprising a differentiated neuronal cell which expresses tyrosine hydroxylase and at least one other indicator of neuronal cell differentiation, said cell having undergone induction ex vivo from an undifferentiated human cell.

13. (amended) A human dopaminergic cell [suitable for transplantation into a human having a dopaminergic deficiency], the cell comprising an ex vivo differentiated human neuronal cell that expresses tyrosine hydroxylase and bcl-2, said cell being capable of synthesizing dopamine and having improved survival [after transplantation].

14. (amended) A method of improving the survival of human neuronal cells [for transplantation], said method comprising the steps of

a. providing a culture of human neuronal cells; and

b. adding a lithium salt to the human neuronal cell culture for a sufficient time to enhance expression of bcl-2[;

c. testing cells from the treated cell culture for the presence of bcl-2;

d. isolating the cells from the culture to produce an isolated cell preparation; and

e. testing the isolated cell preparation for sterility before packaging the cells for transport].

15. (amended) A pharmaceutical dosage form of human non-fetal dopaminergic cells [suitable for transplantation in Parkinson's Disease] comprising

isolated, [purified] neuronal cells, the neuronal cells being capable of expressing tyrosine hydroxylase, D2 dopamine receptor and aldehyde dehydrogenase-2; and

a pharmaceutical diluent.

17. (amended) The method of claim 14 [15] wherein the lithium salt is lithium chloride.

19. (amended) A method of preparing human dopaminergic neuronal cells [suitable for treating Parkinson's disease], the method comprising:

a. providing NT2/D1 cells;

b. culturing NT2/D1 cells with an inducing agent for a time sufficient to optimize tyrosine hydroxylase (TH) expression therein; and

c. replating and culturing the TH-optimized cells in mitotic inhibitor[; and

d. separating the TH-optimized cells from the replate culture].

20. (amended) The method of claim 19, additionally comprising the steps of

d. separating the TH-optimized cells from the replate culture;

e. replating the TH-optimized cells on a confluent feeder cell layer, the cell layer being chosen from cells which stabilize[d] TH production [including bone marrow stem cells, TM4 Sertoli cells, glioma cells, or a combination thereof]; and

f. isolating the TH-optimized cells and stabilized cells from the replate medium.

21. (amended) A pharmaceutical composition [for treating Parkinson's Disease, the composition] comprising

isolated, [purified,] post-mitotic neuronal cells, the neuronal cells expressing tyrosine hydroxylase (TH), D2 dopamine receptor, and aldehyde dehydrogenase-2;

cells capable of stabilizing TH [tyrosine hydroxylase] production of the neuronal cells; and

a pharmaceutical diluent.

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Intrastriatal and Intranigral Grafting of hNT Neurons in the 6-OHDA Rat Model of Parkinson's Disease

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The clinical findings on neural transplantation for Parkinson's disease (PD) reported thus far are promising but many issues must be addressed before neural transplantation can be considered a routine therapeutic option for PD. The future of neural transplantation for the treatment of neurological disorders may rest in the discovery of a suitable alternative cell type for fetal tissue. One such alternative may be neurons derived from a human teratocarcinoma (hNT). hNT neurons have been shown to survive and integrate within the host brain following transplantation and provide functional recovery in animal models of stroke and Huntington's disease. In this study, we describe the transplantation of hNT neurons in the substantia nigra (SN) and striatum of the rat model for PD. Twenty-seven rats were grafted with one of three hNT neuronal products; hNT neurons, hNT-DA neurons, or lithium chloride (LiCl) pretreated hNT-DA neurons. Robust hNT grafts could be seen with anti-neural cell adhesion molecule and anti-neuron-specific enolase immunostaining. Immunostaining for tyrosine hydroxylase (TH) expression revealed no TH-immunoreactive (THir) neurons in any animals with hNT neuronal grafts. THir cells were observed in 43% of animals with hNT-DA neuronal grafts and all animals with LiCl pretreated hNT-DA neuronal grafts (100%). The number of THir neurons in these animals was low and not sufficient to produce significant functional recovery. In summary, this study has demonstrated that hNT neurons survive transplantation and express TH in the striatum and SN. Although hNT neurons are promising as an alternative to fetal tissue and may have potential clinical applications in the future, further improvements in enhancing TH expression are needed. ©2000 Academic Press

Key Words: **hNT neurons; neural transplantation; double transplants; Parkinson's disease; tyrosine hydroxylase; lithium chloride.**

INTRODUCTION

The development of alternatives to fetal-derived cells for use in neural transplantation is of critical importance in the future of transplantation strategies for the treatment of neurodegenerative diseases such as Parkinson's disease (PD). The ideal source of cells for the treatment of PD would provide a limitless supply of dopamine-producing cells capable of reinnervating the host brain without the risk of immunorejection or transmission of infection. Currently, a number of possible cell sources of both neuronal and nonneuronal origin are being studied. Xenografts of porcine-derived fetal ventral mesencephalic (FVM) tissue have been observed to survive in the neostriatum of PD patients for up to 7 months (12). Intrastriatal grafts of fetal porcine tissue in 6-hydroxydopamine (6-OHDA)-lesioned rats have also been shown to survive, provide functional benefit, and reinnervate the host striatum (15, 21, 22). Other researchers have focused on the development of genetically engineered cell lines that overexpress tyrosine hydroxylase (TH) (13, 14, 17, 18, 23, 29, 44, 49, 52, 59) or neurotrophic factors that promote survival of dopaminergic cells (6, 28). Despite these efforts, transplantation of genetically engineered cells in animal models of PD has not provided conclusive long-term beneficial effects or reinnervation of the dopamine-depleted striatum. Another area that is currently being explored by a number of investigators including our own laboratory is the use of neural stem cells, which have the capacity for neuronal differentiation and migration (45). Although there have been reports that transplantation of embryonic-derived stem cell progeny survive, only a limited number of TH-immunopositive cells were identified in the graft (50) suggesting that this alternative is promising but not yet fully developed.

More recently, cell lines of immortalized tumor cells including a human embryonal carcinoma-derived neuronal population (hNT) have been developed. Transplantation of these hNT cells produced behavioral recovery from focal ischemia (7, 8, 48) and quinolinic-acid-induced striatal lesions (19). hNT neurons have also been grafted into rats with experimental brain injury. However, no significant improvement in behavioral recovery was noted (38, 41). hNT neurons are

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Exhibit

derived from a human embryonic carcinoma cell line, NT2/D1 (3). In contrast to other teratocarcinoma cell lines, which are capable of differentiating into neuronal, glial, and mesenchymal phenotypes, the NT2/D1 cells appear to be progenitor cells which have a progeny restricted to the neuronal lineage (called hNT neurons) following retinoic acid (RA) treatment (2–4, 27, 42, 43). The hNT neuronal progeny has been well characterized and it has been shown that these cells closely resemble human neurons (42, 43). Furthermore, the hNT neurons bear glutamate receptor channels (60), produce β-amyloid peptide (10, 30, 55, 57), and express mRNA for glutamic acid decarboxylase, choline acetyltransferase, and D1 and D2 dopamine receptors (19). hNT neurons (24) or their precursor, NT2 cells (36, 37), transplanted into the brains of immunodeficient nude mice survived for over 12 months without evidence of necrosis, apoptosis, graft rejection, or tumor formation. Survival of hNT neurons transplanted into the cerebral cortex and hippocampus of cyclosporine-treated neonatal and adult Sprague–Dawley rats has also been demonstrated (54). These grafts survived for up to 12 weeks and no tumor formation was observed. In a recent study, Konobu and colleagues observed that hNT neurons populated the photoreceptor layer as a stratum following epiretinal injections of the cells at 56 days and suggested that hNT neurons may take on the morphology and function of photoreceptors (26).

The purpose of the present study was to determine whether hNT neurons survive when implanted into the striatum and substantia nigra (SN) of rats with unilateral 6-OHDA lesions of the dopaminergic nigrostriatal pathway and to assess the ability of these neurons to express TH and produce functional effects. We studied three different neuronal products provided by Layton Bioscience, Inc. (Gilroy, CA). The products tested include hNT neurons and two hNT hybrids: hNT-DA neurons and lithium chloride (LiCl) pretreated hNT-DA neurons. hNT neuron cultures are treated with RA acid for 6 weeks and then replated at one-third of the density in the presence of mitotic inhibitors, cytosine arabinoside, and fluorodeoxyuridine for 6 days. hNT-DA neurons are hNT neuron cultures treated with RA for only 4 weeks followed by replating and treatment with the same mitotic inhibitors. A shorter RA treatment time appears to enhance the number of cells expressing TH (personal communication, Mike McGrogan, Layton Bioscience, Inc.). The third product we used was LiCl pretreated hNT-DA neurons. These are hNT-DA neurons in which LiCl was added to the culture for 6 days following replating and mitotic inhibitor treatment (personal communication, Mike McGrogan, Layton Bioscience, Inc.). LiCl has been shown to promote the expression of TH in hNT neurons (62).

MATERIALS AND METHODS

Study Design

A total of 30 female Wistar rats (Charles River, St. Constant Quebec, Canada) were used in this study. All animals received unilateral lesions of the right nigrostriatal pathway with 27 rats later receiving intrastriatal and intranigral grafts (double grafts) of hNT neurons. Three hNT neuronal products (hNT neurons, hNT-DA neurons, LiCl pretreated hNT-DA neurons) were transplanted in this experiment. Sixteen animals received double grafts of hNT neurons, 7 received hNT-DA neurons, 4 received LiCl pretreated hNT-DA neurons, and 3 served as controls and received a lesion only. Functional recovery was assessed by amphetamine-induced rotational behavior.

Animals and 6-OHDA Lesions

Thirty female Wistar rats (Charles River), weighing 200–225 g, were housed 2 animals per cage with food and water *ad libitum* and allowed to acclimatize to the animal care facility for 7 days before surgery. All animal procedures were in accordance with the guidelines of the Canadian Council on Animal Care and the University Council on Laboratory Animals. Rats were anesthetized intramuscularly with 3.0 ml/kg of a ketamine–xylazine–acepromazine anesthetic mixture (25% ketamine hydrochloride; (Ketalean, MTC Pharmaceuticals, Cambridge, Ontario); 6% xylazine; (Rompun, Miles Canada, Etobicoke, Ontario); 2.5% acepromazine maleate; (Wyeth-Ayerst Canada, Montreal, Quebec)) in 0.9% saline and received two stereotactic injections of 6-OHDA (Sigma Chemical Company, Chicago, IL) (3.6 μg of 6-OHDA HBr/μl in 0.2 mg/ml of L-ascorbate in 0.9% saline) into the right ascending mesostriatal dopaminergic pathway at the following coordinates: (1) 2.5 μl at anteroposterior (A/P): −4.0, mediolateral (M/L): −1.2, dorsoventral (D/V): −7.8, toothbar: −2.4; and (2) 3.0 μl of 6-OHDA at A/P: −4.0, M/L: −0.8, D/V: −8.0, toothbar: +3.4. The rate of injection was 1 μl/min with the cannula being left in place for 5 min before being slowly retracted. Animals were allowed to recover for 2 weeks in the animal care facility before being given an amphetamine challenge (5.0 mg/kg, ip) and their rotational scores were collected over a 70-min period using a computerized video activity monitor programmed for rotational behavior (Videomex, Columbus Instruments, Columbus, OH). Only animals exhibiting a mean ipsilateral rotational score of eight or more complete full body turns per minute were included in the study. Animals were tested for rotational behavior at 3 and 6 weeks post-transplantation. Statistical analysis for between-group and within-group differences was assessed at $P < 0.05$ using a two-way ANOVA followed by Tukey's post hoc test.

TABLE 1

The Mean (±Standard Deviation) Viability of hNT Neurons and the Number of Animals Transplanted with hNT, hNT-DA, and LiCl Pretreated hNT-DA Neurons

hNT neuronal product	Animals grafted	Viability	Total cells implanted
hNT	16	50.2 (±6.8)	~800,000
hNT-DA	7	47.8 (±9.1)	~800,000
LiCl pretreated hNT-DA	4	50.5 (±2.9)	~800,000

Preparation and Transplantation of hNT Cell Suspensions

The frozen hNT neurons were obtained from Layton Bioscience (hNT neurons, hNT-DA neurons, and LiCl pretreated hNT-DA neurons) and stored at −80°C until the time of transplantation. Two weeks following 6-OHDA lesions, rats were chosen for transplantation if they exhibited a mean rotational score of eight full body turns per minute. Beginning on the day of surgery, each animal received 10 mg of cyclosporin A/kg body wt ip for the duration of the experiment. Prior to transplantation, the hNT neurons were quickly thawed by placing them in a water bath at 37°C. The cells were then washed three times in DMEM/0.05% DNase (Sigma Chemical Company). The cells were suspended and the cell viability and suspension concentration calculated. The trypan blue dye exclusion method, which stains dead cells blue and fails to stain live cells, was used to assess cell viability (Table 1).

The cell suspensions were stereotactically injected both intrastriatally and intranigrally using a technique previously described (33, 35). A specially designed capillary tip micropipette with an outer opening diameter of 50–70 μm is attached to a 2-μl Hamilton syringe and used to sterotatically implant the desired number of cells at a rate of 100 nl/min into both the SN and the striatum (400,000 cells/site). Each animal received a total of about 800,000 cells. Injection of the cells into the dorsolateral striatum occurs at the following coordinates: (1) A/P: +1.3, M/L: −2.1, D/V: −5.5 and −4.3; (2) A/P: +0.6, M/L: −2.9, D/V: −5.5 and −4.3; and (3) A/P: +0.3, M/L: −3.7, D/V: −5.5 and −4.3; toothbar: −3.3; coordinates from Bregma and dorsal surface of the skull and the SN at the following coordinates: (1) A/P: −4.8, M/L: −2.0, D/V: −8.3 and −8.1; (2) A/P: −5.0, M/L = −2.3, D/V = −8.2 and −8.0; and (3) A/P: −5.3, M/L: −2.6, D/V: −8.1 and −7.9; toothbar: −3.3; coordinates from Bregma and the dorsal surface of the skull.

Immunohistochemistry

At about 6 weeks posttransplantation, the rats were euthanized with an overdose of a ketamine–xylazine–acepromazine mixture and perfused transcardially with 100 ml of 0.1 M phosphate buffer (PB) followed by 250 ml of 4% paraformaldehyde in 0.1 M PB for 10 min. The brains were then removed from the cranium to be postfixed with 4% paraformaldehyde in 0.1 M PB overnight before being stored for 24 h in phosphate-buffered saline containing 30% sucrose. With the freezing microtome, 40-μm coronal sections were cut and stored in Millonig's solution (6% sodium azide in 0.1 M PB) until immunohistochemical processing of the sections could be performed. Following processing, sections were mounted in 0.1 M PB on gelatin-coated slides and coverslipped with permount. Estimates of surviving cell numbers were calculated in every fourth section through the graft (6–10 sections per animal) using Abercrombie's formula (1). The cell diameter used in the calculations for the Abercrombie's formula was 14 μm, which was the average diameter measured of the TH-immunoreactive (THir) cells. All data were analyzed for between-group and within-group differences at $P < 0.05$ using a two-way ANOVA followed by Tukey's post hoc test.

Tyrosine hydroxylase. Staining for the presence of TH was performed using the primary rabbit anti-TH antibody (Ab;1:2500 Pel Freeze Biologicals, Rogers, AR) and the ABC-kit (Vector Laboratories Canada, Inc., Burlington, Ontario, Canada). For this procedure the sections were prewashed for 10 min in a solution of 10% methanol and 3% hydrogen peroxide and blocked in PB containing 0.3% Triton X-100 and 5% NSS for 1 h. The sections were removed and incubated in a 1:2500 solution of rabbit polyclonal anti-TH Ab for 16 h. To visualize Ab binding, 1:500 biotinylated swine anti-rabbit IgG Ab (Dako Diagnostics Canada, Inc., Mississauga, Ontario, Canada) was used followed by a streptavidin-biotinylated HRP complex kit. The peroxidase activity was visualized by the addition of 3,3'-diaminobenzidine (DAB). The sections were then washed in 0.1 M PB before being mounted.

Human neural cell adhesion molecule. Staining for the presence of neural cell adhesion molecule (NCAM) was performed using the primary mouse anti-human NCAM monoclonal antibody (MOC1; 1:1000 DAKO Diagnostics Canada, Inc.) and the ABC kit. Briefly, the sections were prewashed for 30 min in a solution of 10% methanol and 3% hydrogen peroxide and blocked in PB containing 0.3% Triton X-100 and 5% normal horse serum for 1 h. The sections were removed and incubated in a 1:1000 solution of monoclonal mouse anti-NCAM (MOC1) Ab for 16 h. To visualize Ab binding, 1:250 biotinylated horse anti-mouse IgG Ab (Vector Laboratories Canada, Inc.) was used followed by a streptavidin-biotinylated HRP complex kit. The peroxidase activity was visualized by the addition of DAB.

Human neuron-specific enolase. Staining for the presence of human-neuron-specific enolase (NSE) was performed using the primary mouse anti-NSE mono-

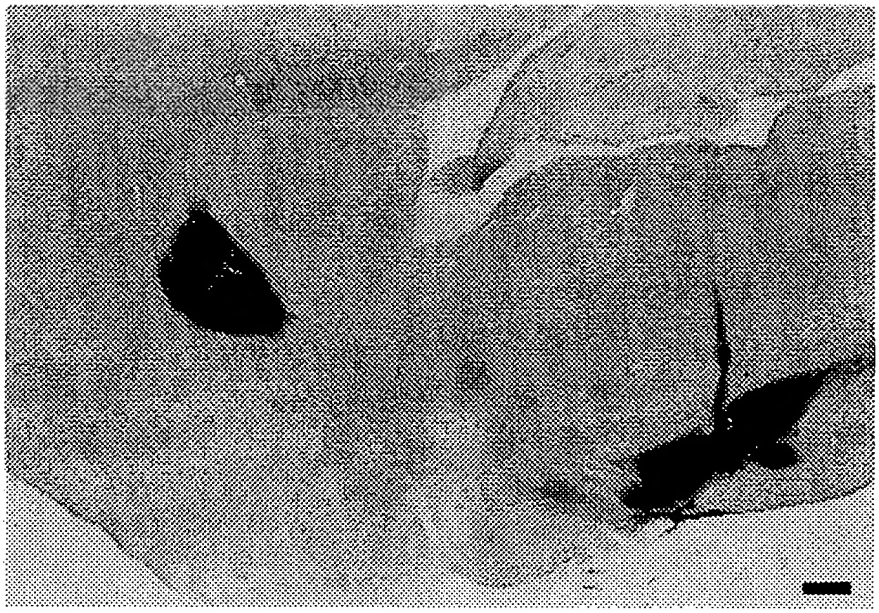

FIG. 1. Representative parasagittal section through a double hNT grafted rat brain immunostained for human NSE (scale bar = 300 μm).

clonal antibody (1:100; Vector Laboratories Canada, Inc.) and the ABC kit. The sections were prewashed for 30 min in a solution of 10% methanol and 3% hydrogen peroxide and blocked in PB containing 0.3% Triton X-100 and 5% NHS for 1 h. The sections were removed and incubated in a 1:100 solution of mouse monoclonal anti-hNSE Ab for 16 h. To visualize Ab binding, 1:200 biotinylated horse anti-mouse IgG Ab was used followed by a streptavidin-biotinylated HRP complex kit and DAB.

RESULTS

Survival of hNT Neuronal Grafts

All animals that received both intrastriatal and intranigral grafts of the hNT neuronal products (Fig. 1) had surviving grafts that were strongly immunostained for the presence of both human NSE (Figs. 2A, 2B, 3A, and 3B) and human NCAM (Figs. 2C, 2D, 3C, and 3D). Analysis of the hNT grafts by anti-NCAM immunohistochemistry (Figs. 2C, 2D, 3C, and 3D) revealed a strong staining of the entire graft area and darkly stained cell-like structures could clearly be seen within the graft boundary. The overall strong immunostaining of the graft made the determination of cell numbers impossible. NCAMir fibers extending beyond the graft–host interface could be seen in many of the grafted animals. NSE immunohistochemistry (Figs. 2A, 2B, 3A, and 3B) produced a similar strong staining pattern, with what appeared to be more darkly stained cells within the graft, but again counts could not be accurately determined. NSEir fibers were seen extending beyond the graft–host interface at the level of the striatum and, in some cases, fibers were observed to extend greater than 100 μm into the surrounding host tissue.

Expression of TH by hNT Neurons

Analysis of TH expression in animals with hNT neuron grafts (n = 16) showed no THir cells in either the striatum or the SN (Figs. 2E and 2F). In 43% of animals with grafts of hNT-DA neurons (n = 3), readily identifiable THir cells within both the striatum and the SN were observed. THir neurons appeared healthy and had processes extending for variable distances in the host brain. However, fiber outgrowth was sparse both within the graft and in the host tissue surrounding the graft. In these animals, there were 435.12 ± 323.30 THir cells within the striatum and 393.68 ± 204.70 within the SN (Fig. 5). THir cells were observed in 100% of animals with intrastriatal and intranigral grafts of LiCl pretreated hNT-DA neurons (Fig. 4). The mean (±SD) number of THir cells within the intrastriatal and intranigral grafts was 489.39 ± 18.09 and 319.68 ± 142.08, respectively (Fig. 5). There was no significant difference in the number of THir neurons between the hNT-DA neuronal and LiCl pretreated hNT-DA neuronal grafts ($P > 0.05$). Similarly, there was no significant difference in the number of THir cells between the intrastriatal and intranigral graft locations ($P > 0.05$).

Amphetamine-Induced Rotational Behavior

There was not a statistically significant reduction in amphetamine-induced rotational behavior at any of

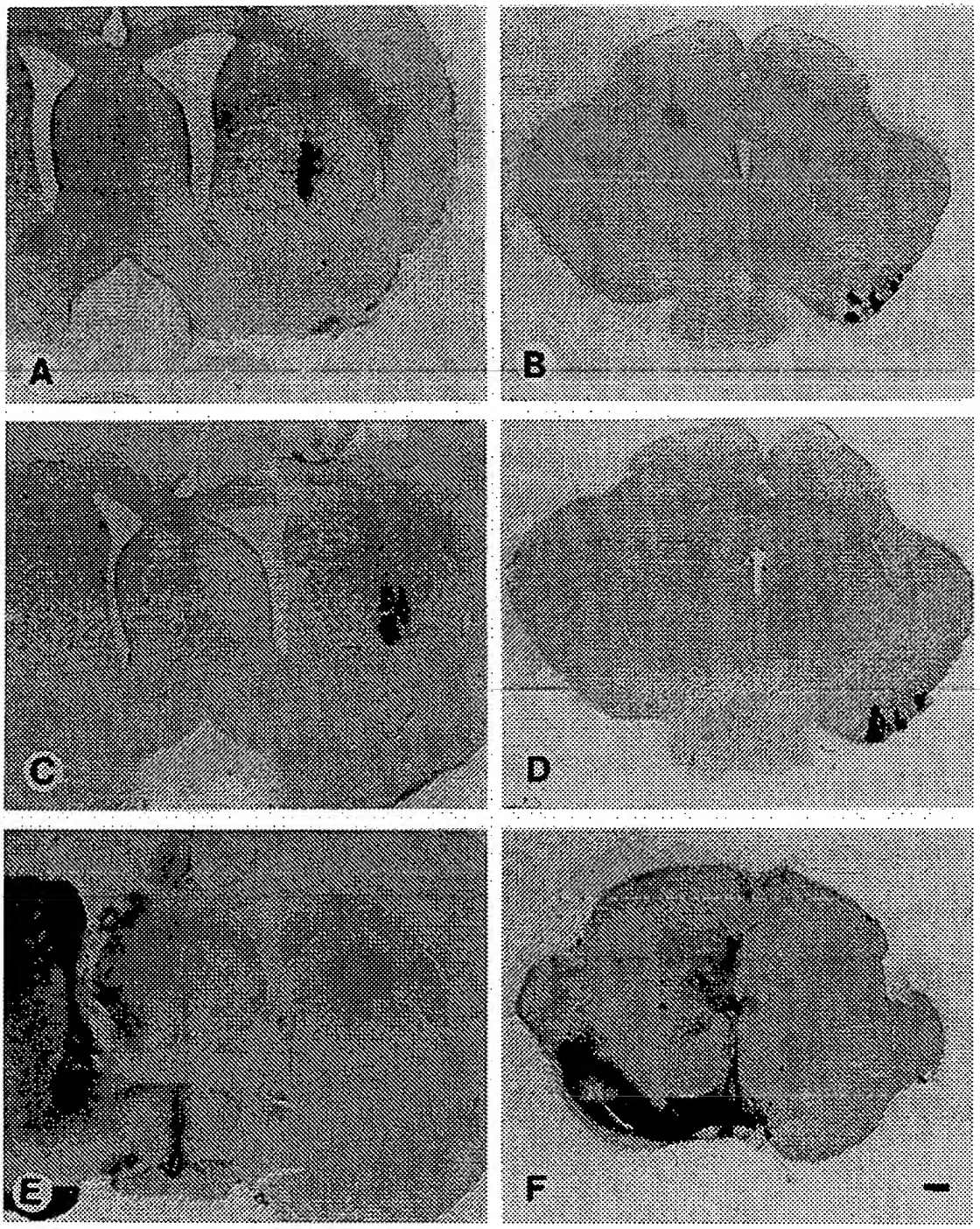

FIG. 2. Representative coronal sections through the level of the striatum and substantia nigra of rats with double grafts of hNT neurons immunostained for the presence of NSE (A and B). Adjacent sections were stained for the presence of NCAM (C and D) and TH (E and F). Although grafts are visualized following immunostaining for anti-NSE and -NCAM, note the absence of THir profiles in the grafted striatum and nigra (scale bar = 250 μm).

the time points tested regardless of the product implanted. In animals maintained for 6 weeks with double grafts of either hNT-DA neurons or LiCl pretreated hNT-DA neurons rotational behavior exhibited a trend toward decreasing rotations, but this did not reach statistical significance. There was a correlation between surviving THir cells and rotational scores. Only animals that had surviving THir cells (43% of the hNT-DA group and the LiCl pretreated hNT-DA group) had decreased rotational scores while animals with no

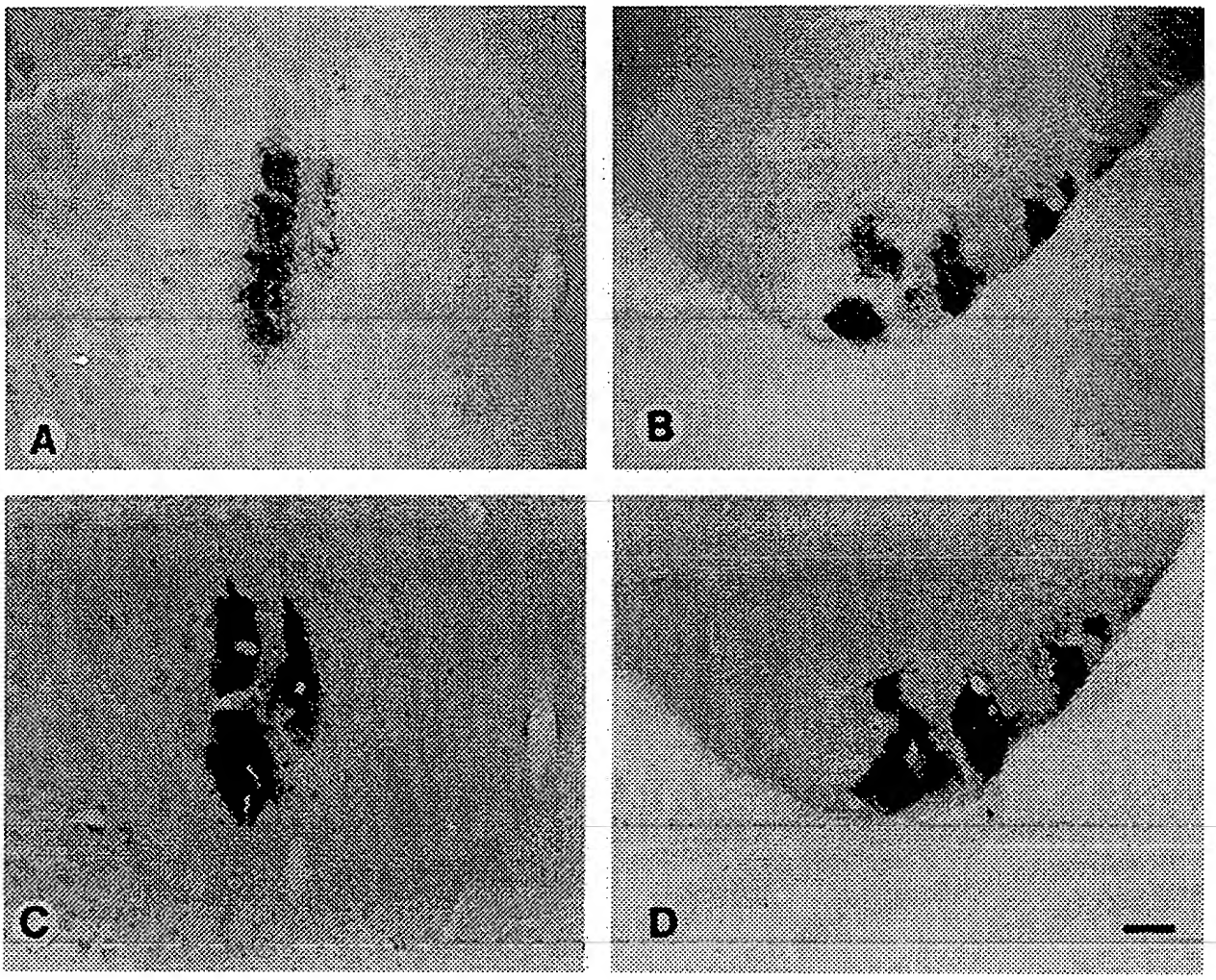

FIG. 3. Higher power photomicrographs of intrastriatal and intranigral hNT neuronal grafts immunostained for human NSE (A and B) and NCAM (C and D). Note the dark staining of the graft that made counts of the number of surviving cells impossible (scale bar = 150 μm).

THir cells (hNT-neuronal grafts and lesion only groups) did not exhibit any reduction in mean full body turns (Fig. 6).

DISCUSSION

Graft Survival

Immunostaining with anti-NCAM demonstrated that hNT neurons survive the transplantation procedure. This observation is in agreement with previous studies demonstrating survival of hNT neurons *in vivo* by using anti-NCAM immunohistochemistry (7, 8, 24, 36–38, 41, 48, 54). In addition, we have shown that hNT grafts can also be visualized with antibodies recognizing human NSE, which reflects the human origin of hNT neurons, originally derived from a human teratocarcinoma. TH immunohistochemistry of grafted hNT neurons demonstrated that the 6-week cultured hNT neurons fail to express TH, whereas in 43% of the animals receiving hNT-DA and 100% of animals with LiCl pretreated hNT-DA neuronal grafts there was evidence of THir neurons. The reason for TH expression in only 43% of animals receiving hNT-DA neuronal grafts is unknown but it is possible that these grafts may need time to mature and long-term studies are currently underway in our laboratory to address this issue. It is also possible that these cells need an additional factor such as LiCl to promote differentiation into a TH-expressing neuronal phenotype (62). This concept is supported by the observation that 100% of animals grafted with hNT-DA neurons pretreated with LiCl had surviving THir neurons.

All of the animals with grafts of LiCl pretreated hNT-DA neurons exhibited THir cells within the grafts in the present study. A recent study reported that TH expression in hNT neurons was increased sixfold *in vitro* following 5 days of exposure to LiCl (62). Previous studies have also shown that *in vitro* lithium treat-

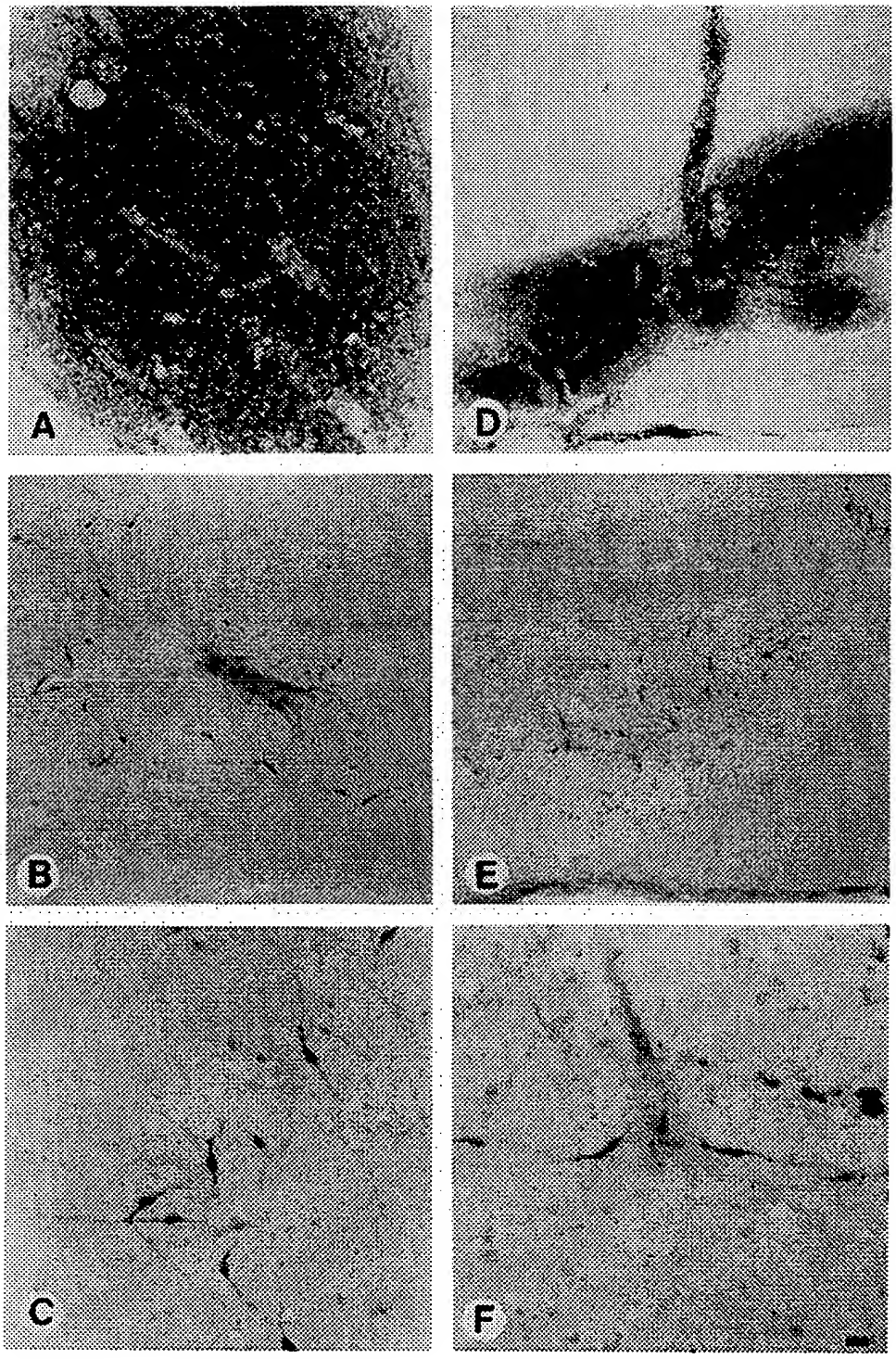

FIG. 4. Intrastriatal (A–C) and intranigral (D–F) LiCl-treated hNT-DA neuron grafts immunostained for NSE (A and D). Adjacent sections were immunostained for the presence of TH (B and E). C and F are higher power photomicrographs of B and E. Note that THir fibers can be seen extending from the cell bodies (scale bar = (A and D) 250 μm; (B and E) 500 μm; (C and F) 1000 μm).

ment increased the expression of TH in SH-SY5Y neuroblastoma cells (9) and bovine adrenal medullary cells (51). Other strategies to enhance TH expression have also been used. Small increases in TH expression could be obtained when hNT neurons were cultured in the presence of acidic fibroblast growth factor, protein kinase pathway activators, and other coactivators (20). Furthermore, Othberg and colleagues have also dem-

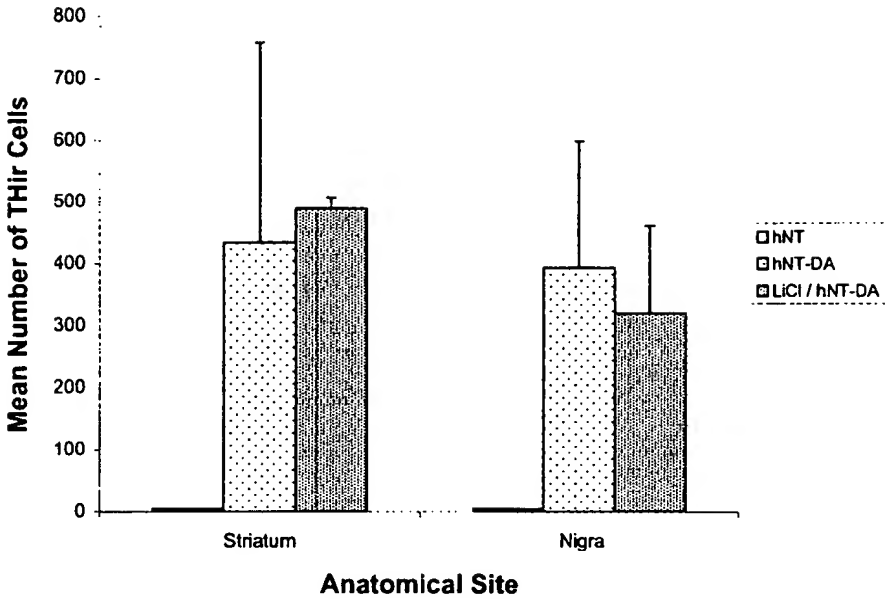

FIG. 5. Bar graph demonstrating the mean (±SD) THir cells found within the intrastriatal and intranigral grafts of hNT neurons, hNT-DA neurons, and LiCl pretreated hNT-DA neurons. No significant difference in the number of cells was observed between the striatal or nigral location of the grafts. No surviving THir cells were encountered in animals grafted with hNT neurons. There was no significant difference in the number of THir neurons in rats grafted with hNT-DA neurons or LiCl pretreated hNT-DA neurons. However, only 43% of animals with hNT-DA neuron grafts contained THir neurons.

onstrated a greater enhancement of TH expression by hNT neurons when cocultured with porcine Sertoli cells (40). Although these studies demonstrate an enhancement of TH expression in hNT neurons *in vitro,* it has yet to be determined whether these cells continue to express TH *in vivo.* There is also evidence that the hNT precursors, NT2 cells, are capable of transfection with foreign genes (25, 53) and hNT neurons are readily infected by vaccinia viruses (11), suggesting alternative methods for enhancing TH expression in these cells.

Interestingly, the number of surviving THir neurons was not different when transplanted in either the striatum or the SN. This suggests that the homotopic site

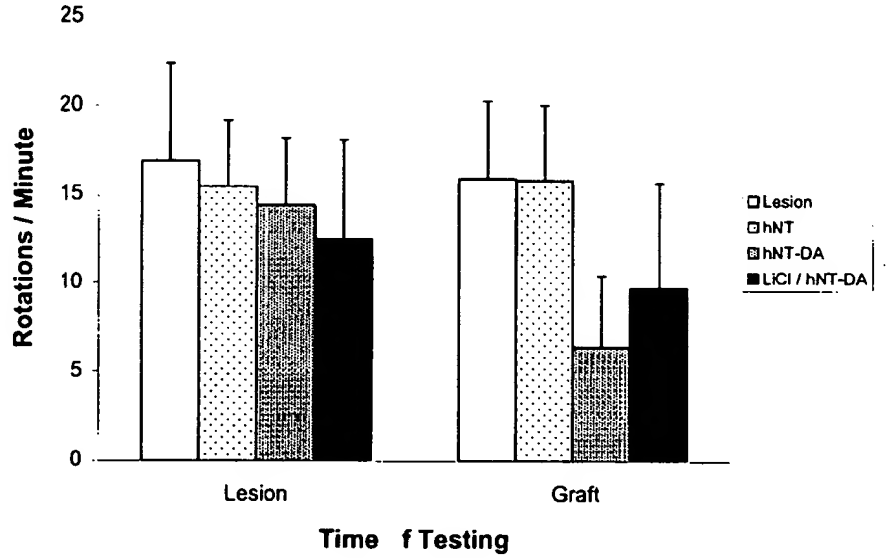

FIG. 6. The mean (±SD) rotations per minute with amphetamine challenge (5 mg/kg, ip), following 6-OHDA-induced lesions of the right ascending dopaminergic nigrostriatal pathway and 6 weeks following double grafting of hNT neurons, hNT-DA neurons, and LiCl pretreated hNT-DA neurons. A control group of lesion only animals is also included. Although a reduction of rotational behavior was observed in the hNT-DA neuron and LiCl pretreated hNT-DA neuron groups, this reduction did not achieve statistical significance.

(SN) environment does not influence the phenotype of hNT neurons. It has been reported that the mouse caudoputamen may influence the differentiation of hNT neurons into a dopaminergic phenotype (37); however, our study does not provide evidence that the rat SN may influence the hNT neurons to differentiate into TH neurons to a greater extent when compared to the striatum.

The failure of hNT neurons to provide functional recovery in the present study may relate to the relatively low number of THir neurons and poor fiber outgrowth observed in surviving grafts. Fiber outgrowth and number of surviving THir neurons strongly correlate with the extent of functional recovery in fetal grafts (5, 46). It is possible that hNT cells mature at a slower pace than fetal dopaminergic neurons and long-term studies may be necessary to test this hypothesis.

hNT Neurons as an Alternative Tissue Source for Neural Transplantation?

The optimal cell for transplantation in Parkinsonian patients would be one that is not only abundant and readily available but also has the capability of synthesizing dopamine and reinnervating the nigrostriatal dopaminergic system. hNT neurons have some of these qualifications; they are readily available and able to proliferate in culture (2–4, 27, 42, 43). There is evidence that hNT neurons can survive transplantation into the adult rodent brain (24, 54). Reversal to their neoplastic phenotype has not been observed and the present study has shown that hNT neurons survive transplantation into the striatum and SN, integrate into the host, and express TH.

Although we have not shown that hNT neurons are capable of releasing dopamine, there is evidence that hNT neurons are immunopositive for markers of secretory activity *in vitro* (43). However, dopamine production may not be enough for functional restoration in PD. It is well known that grafting various cell lines transfected with the tyrosine hydroxylase gene reduces dopamine agonist-induced behavioral deficits in the Parkinson rat model (13, 14, 17, 18, 23, 29, 44, 49, 52, 59). However, reinnervation of the host brain may also be crucial for restoring complex sensorimotor deficits in lesioned animals (31, 32, 34, 39, 46). hNT neurons may have the capability of producing and secreting dopamine and also reinnervating the host. This concept is supported by our observation that hNT neurons express TH after implantation and extend processes into the host brain. Further enhancement of host reinnervation could be accomplished by increasing the differentiation of hNT neurons into THir cells and promoting their fiber outgrowth. Our laboratory and several other investigators have demonstrated increased fiber outgrowth of dopaminergic transplants using glial cell-line-derived neurotrophic factor (GDNF) (5, 16, 31, 47, 56, 58) and brain-derived neurotrophic factor (BDNF) (61). It is possible that the addition of neurotrophic factors such as GDNF or BDNF to hNT neurons may similarly increase survival of THir neurons and induce fiber outgrowth.

Concluding Remarks

This study has demonstrated that hNT neurons survive implantation, integrate into the host brain, and express TH when grafted into the striatum or SN. Although THir neurons were found in the striatum and SN, the numbers were relatively small and expression of a TH phenotype appeared to be independent of the site of implantation (striatum versus nigra). This study has also provided evidence that LiCl treatment may be beneficial in enhancing TH expression of hNT neurons.

hNT neurons are promising as a possible alternative to fetal tissue for transplantation in animal models of PD and may have potential clinical applications in the future. However, before hNT neurons can be considered a reliable cell source in experimental neural transplantation for PD, further improvements in enhancing TH expression are needed.

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